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Short communication

Bovine lactoferrin and lactoferricin interfere with intracellular trafficking of Herpes simplex virus-1

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Abstract

Although both lactoferrin (Lf), a component of the innate immune system of living organisms, and its N-terminal pepsin cleavage product lactoferricin (Lfcin) have anti-herpes activity, the precise mechanisms by which Lf and Lfcin bring about inhibition of herpes infections are not fully understood. In the present study, experiments were carried out to characterize the activity of bovine Lf and Lfcin (BLf and BLfcin) against the Herpes simplex virus-1 (HSV-1). HSV-1 cellular uptake and intracellular trafficking were studied by immunofluorescence microscopy. In comparison to the untreated infected control cells, both the BLf- and BLfcin-treated cells showed a significant reduction in HSV-1 cellular uptake. The few virus particles that were internalized appeared to have a delayed intracellular trafficking. Thus, in addition to their interference with the uptake of the virus into host cells, Lf and Lfcin also exert their antiviral effect intracellularly.

Keywords: Lactoferrin; Lactoferricin; Herpes virus; HSV-1; Antiviral

1. Introduction

Herpes simplex virus-1 (HSV-1) causes a wide spectrum of mild to severe disorders and is one of the most widespread agents infecting people of all ages [1]. HSV-1 is an enveloped DNA virus which enters its host cells through fusion of the viral envelope with the plasma membrane. The incoming capsids are released in the cytoplasm and efficiently transported along microtubules to the nuclear pore complexes, where the viral DNA genomes are released into the nucleus by an unknown mechanism [2]. Therefore, interference with the early stages of the virus life cycle is a good strategy to employ in the development of new anti-herpes drugs.

A new class of clinically useful antiviral compounds comprises cationic proteins and peptides produced by almost all species of life as part of their immediate non-specific defense against infections. Of these, bovine lactoferrin (BLf) and its N-terminal pepsin cleavage product lactoferricin (BLfcin) have *in vitro* antiviral activity against a wide range of human and animal viruses (reviewed in Ref. [3]), including HSV-1 [4–9]. The precise mechanisms by which Lf and Lfcin exert their antiviral effect remain largely elusive, however. It has been demonstrated that Lf inhibits adsorption of HSV-1 by the host cells due to its affinity for heparan sulfate and other glycosaminoglycans [8,10], which are typical HSV-1 attachment receptors on the cell membrane. A binding site for these carbohydrates is found in Lfcin, and both Lf and Lfcin have demonstrated high affinity for heparan sulfate proteoglycans [11–13]. Thus, it has been speculated that Lfcin also interacts with these carbohydrates to block HSV-1 cellular entry. Recently, it was shown that in addition to inhibiting viral adsorption, BLf also targets the HSV-1 entry process by interacting with the structural viral proteins ICP-5 (major capsid protein) and VP-16 (viral tegument protein) [14], and inhibiting the cell-to-cell viral spread [15]. This data indicates that Lf and Lfcin exert their antiviral effects at different stages of the HSV-1 life cycle. Here, we investigate the effects of Lf and Lfcin in the cellular uptake and intracellular trafficking of HSV-1.

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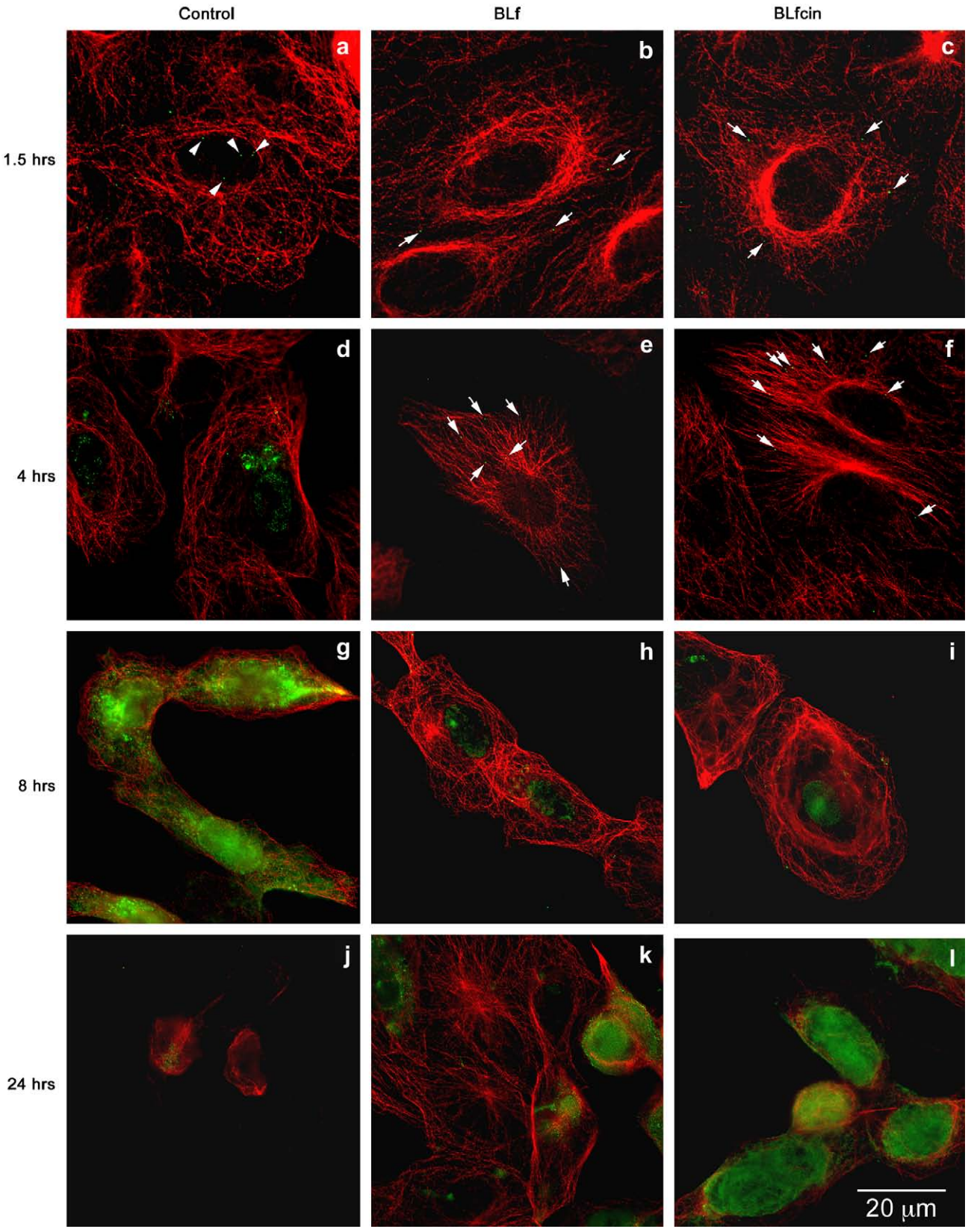

Fig. 1. BLf and BLfcin reduction of HSV-1 uptake and delay of the trafficking of HSV-1 towards the nucleus. Immunofluorescence microscopy of Vero cells infected with HSV-1 in the absence of BLf/BLfcin (Control; a, d, g, j), or in the presence of BLf (0.6 μM; b, e, h, k) and BLfcin (14.6 μm; c, f, i, l). Cells were fixed at 1.5, 4, 8, or 24 h after infection, and labeled with antibodies against tubulin (red) and HSV-1 capsid proteins (green). The arrows indicate the few HSV-1 capsids that entered the cells treated with BLf/BLfcin at 1.5 and 4 h post-infection. The arrowheads in (a) point to the viral proteins already accumulating in the nucleus at 1.5 h post-infection. Images shown are representative of three independent experiments performed for each condition.

2. Materials and methods

2.1. Cells, virus and reagents

Vero cells were maintained at 5% CO_2 and 37 °C in Minimum Essential Medium (MEM, HyClone), supplemented with 10% fetal bovine serum (Sigma). A stock of HSV-1 (MacIntyre stain, ATCC# VR-539), kindly provided by Dr. David Lawrence (British Columbia Centre for Disease Control, Vancouver, Canada), was obtained by infecting Vero cells at a multiplicity of infection (m.o.i.) of 0.5, and incubating the cells for 2.5 days at 37 °C. Cell debris was then separated by centrifugation at 27,000*g*, and the titre of the virus-containing supernatant was assessed by a plaque assay using standard protocol [2]. BLf with a purity of 90% was from Sigma. BLfcin with a purity of >95% was kindly provided by Dr. Tore J. Gutteberg (University Hospital of North Norway).

2.2. Infection, BLf/BLfcin treatment and immunofluorescence microscopy

Vero cells were grown on glass coverslips to ~70% confluency, and pre-incubated for 10 min at 4 °C with IC_{50} concentrations of either BLf (0.6 μM), BLfcin (14.6 μM) (IC_{50} values were according to Ref. [5]), or medium as a negative control. Subsequently, the cells were infected with HSV-1 at an m.o.i. of 25, and incubated at 4 °C for 1 h, in order to synchronize the viral uptake. Cells were then incubated for 1.5, 4, 8, or 24 h at 37 °C. At each interval, cells were prepared for immunofluorescence microscopy as previously described [2]. The primary antibodies used against HSV-1 and microtubules were the polyclonal rabbit B0114 (DakoCytomation) and the mouse monoclonal anti-alpha tubulin DM1A (Sigma), respectively. The secondary antibodies used were goat anti-mouse rhodamine and goat anti-rabbit fluorescein (Invitrogen). Cells were observed on a Zeiss Axioplan 2 microscope (Carl Zeiss). Immunofluorescence images were obtained from three independent experiments for each condition. To quantify the viral uptake and intracellular trafficking, small, intensely fluorescent-labeled spots corresponding to HSV-1 capsids were counted for the 90-min infection experiments.

In order to detect intracellular localization of BLf and BLfcin, these were biotinylated using the Lightning-Link™ Biotin Conjugation Kit (Innova Biosciences) according to the manufacturer's recommendations. Vero cells were grown on coverslips to ~70% confluency and incubated for 30 min, 1 h or 4 h at 37 °C with biotinylated BLf (0.6 μM) or biotinylated BLfcin (14.6 μM). After incubation, the cells were prepared for immunofluorescence microscopy as described above. Microtubules were immunolabeled, as described above, and the biotinylated protein and peptide were detected with streptavidin-fluorescein (Vector laboratories).

3. Results and discussion

It has been previously surmised that BLf and BLfcin mainly inhibit the attachment of HSV-1 to the host cells through competitive interaction with cell surface heparan sulfate [8,10,16]. There are, however, indications that BLfcin also has anti-HSV activity intracellularly, after the initial binding of the virus to the host cell [10,14]. To investigate whether steps of the HSV-1 infection cycle are altered in the presence of BLf or BLfcin, we followed the cellular uptake and intracellular trafficking of HSV-1 in Vero cells that were treated with BLf or BLfcin, and used immunofluorescence microscopy to visualize both intracellular HSV-1 capsids and host cell microtubules. To enable visualization of the capsids by fluorescence microscopy, the viral load had to be artificially high (m.o.i. of 25). Despite the high viral concentration, both BLf and BLfcin were able to significantly reduce the number of HSV-1 capsids entering the cells, compared to the untreated infection control cells (Fig. 1). HSV-1 cellular uptake was not completely inhibited by the presence of either BLf or BLfcin, however. In the control-infected cells, HSV-1 proteins were already found in the nucleus 1.5 h after viral infection (Fig. 1a). For cells treated with BLf or BLfcin, however, the HSV-1 capsids appeared to remain associated with microtubules at the periphery of the cells (Fig. 1b and c), indicating a possible interference with trafficking of the HSV-1 capsids along microtubules towards the nucleus. Quantification of the number of intensely fluorescent-labeled spots (corresponding to HSV-1 cytoplasmic capsids, or to viral proteins within the nucleus) in 54 cells per condition revealed that only a few fluorescent spots were found in the nuclei of cells treated with BLf or BLfcin 1.5 h post-infection (Table 1). Thus, HSV-1 capsid trafficking and viral protein accumulation within the nucleus appeared to be delayed in the presence of BLf and BLfcin. The delay should not be attributed to the reduction of viral uptake, because control cells infected at an m.o.i. of 2.5, which have numbers of internalized virus capsids similar to that shown in Fig. 1b and c, revealed a larger numbers of fluorescent-labeled spots in the nucleus than the BLf- or BLfcin-treated cells infected at an m.o.i. of 25 (data not shown).

Since only a very few fluorescent-labeled spots were found in the nuclei of BLf- or BLfcin-treated cells 90 min post-infection, we followed HSV-1 infection in the presence and absence of BLf/BLfcin over a more extended course of experiment including 4, 8, and 24 h post-infection times. Most of the HSV-1 capsids were detected in the cytoplasm along microtubules in the BLf- and BLfcin-treated cells at 4 h post-infection (Fig. 1e and f), while in the control-infected cells viral protein had already accumulated within the nucleus within the same time frame (Fig. 1d). At 8 h post-infection, virus proteins had reached the nucleus in the BLf- and BLfcin-treated cells

Table 1
Intracellular fluorescent-labeled spots 90 min post-infection

	Cell periphery	Cytoplasm	Nucleus
Control	7.1%	44.8%	47.6%
BLf	36.7%	62.3%	1.0%
BLfcin	29.7%	67%	3.3%

Values are the percentage of the total number of intensely fluorescently labeled spots found within 54 cells per each condition. Experiments were performed as indicated in Fig. 1.

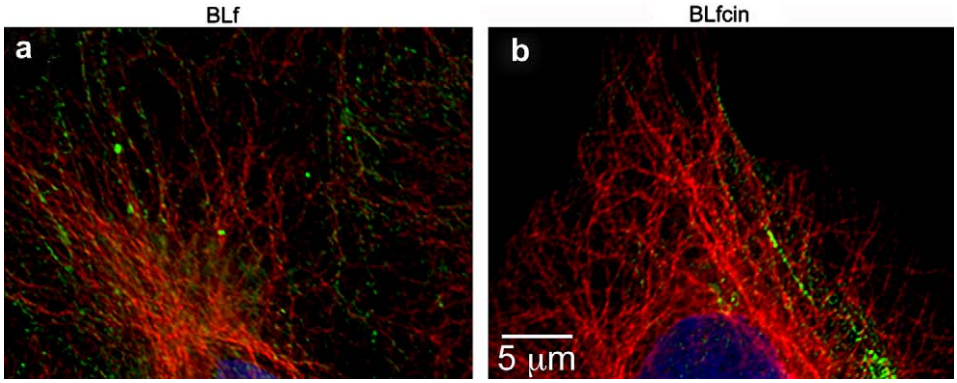

Fig. 2. Biotinylated BLf and BLfcin localization along microtubules. Immunofluorescence microscopy of Vero cells incubated with (a) biotinylated BLf (0.6 μM) and (b) biotinylated BLfcin (14.6 μM) for 30 min, fixed and labeled with an antibody against tubulin (red) and fluorescein-streptavidin (green). Images shown are representative of three independent experiments.

(Fig. 1h and i), indicating that the infection process was delayed by 4 h. It is worth noting, however, that HSV-1 capsids did not accumulate at the nuclear envelope. Thus, BLf and BLfcin apparently did not inhibit nuclear import. At the same time interval, the control-infected cells started to round up, and newly synthesized viral proteins were found throughout the entire cell (Fig. 1g). The control-infected cells did not survive at 24 h post-infection (Fig. 1j), while the BLf- and BLfcin-treated cells had started to round up and were filled with viral proteins (Fig. 1k and l), a stage that the control cells reached at only 8 h post-infection. These results strengthen our observations that intracellular HSV-1 trafficking is apparently delayed in the presence of BLf or BLfcin, consequently delaying viral protein synthesis.

Previously, studies established that Lf can be internalized by other cells such as human monocytes and lymphoblastic like cell lines [17]. To date, the pathway of Lf intracellular uptake is not well understood; thus we have started to analyze this pathway by following the cellular uptake of biotinylated BLf or BLfcin. As illustrated in Fig. 2, Vero cells incubated with biotinylated BLf or BLfcin gave a fluorescent-labeled pattern reminiscent of that of the microtubule network. Negative control experiments of incubating the cells with fluorescein-labeled streptavidin did not yield any fluorescent signal within the cells (data not shown). Our findings are consistent with the uptake of biotinylated Lf/Lfcin by endocytosis, and transport of the Lf/Lfcin filled endosomes along microtubules towards the nucleus periphery. In this way, the trafficking of LF/Lfcin filled endosomes may compete with the microtubule-mediated transport of HSV-1, resulting in a delay in the intracellular trafficking of HSV-1. Moreover, these observations may be an essential component of the antiviral activity of BLf and BLfcin, since intact microtubules play a crucial role in many viral replication mechanisms. Indeed, the antiviral activity of Lf has been demonstrated in several viruses, including the Hepatitis C virus, Rotavirus, Poliovirus, Respiratory syncytial virus, Papillomavirus, HIV, and Cytomegalovirus [3]. For all of these viruses, it has been shown that an intact microtubule network is needed for successful viral replication [18–24].

4. Conclusion

The aim of the present study was to investigate the antiviral effects of BLf and BLfcin on the HSV-1 life cycle within Vero cells. Using indirect immunofluorescence microscopy, we followed the HSV-1 uptake and transport towards the nucleus of host cells in the presence and absence of BLf or BLfcin. Our results demonstrate that, in addition to interfering with viral entry, BLf and BLfcin interfere with viral trafficking along the microtubules, thus delaying viral trafficking towards the nucleus and HSV-1 replication. These observations may reflect an essential mode of antiviral activity of BLf and BLfcin, since intact microtubules play a crucial role in the efficient nuclear targeting of several viruses.

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